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# Bacterial cell motility of *Burkholderia* gut symbiont is required to colonize the insect gut

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ABSTRACT

**We generated a *Burkholderia* mutant, which is deficient of an *N*-acetylmuramyl-L-alanine amidase, AmiC, involved in peptidoglycan degradation. When non-motile Δ*amiC* mutant *Burkholderia* cells harboring chain form were orally administered to *Riptortus* insects, Δ*amiC* mutant cells were unable to establish symbiotic association. But, Δ*amiC* mutant complemented with *amiC* gene restored in vivo symbiotic association. Δ*amiC* mutant cultured in minimal medium restored their motility with single-celled morphology. When Δ*amiC* mutant cells harboring single-celled morphology were administered to the host insect, this mutant established normal symbiotic association, suggesting that bacterial motility is essential for the successful symbiosis between host insect and *Burkholderia* symbiont.**



## 1. Introduction

Insects occupy over one million species and represent the most diverse animal group in the terrestrial ecosystem [1]. Notably, many insects harbor symbiotic microorganisms of mutualistic nature within their gut lumen, body cavity, or cells, and they are often benefited from the symbiosis for their growth, viability and fecundity. Therefore, several insect model systems have been developed to understand the molecular mechanisms of host–microbe interactions [1]. The bean bug *Riptortus pedestris* is known as a pest of leguminous crop in eastern Asia [2] and a member of the stinkbug family Alydidae in the insect order Hemiptera [3]. In contrast to the previously known insect–bacteria symbiotic systems, nymphal *R. pedestris* acquires a beta-proteobacterial symbiont of the genus *Burkholderia* from the soil environment every generation [4]. *R. pedestris* possesses a symbiotic organ, the posterior midgut, containing numerous crypts filled with bacterial cells of the symbiotic *Burkholderia*. The symbiotic *Burkholderia* cells are easily cultivable on standard culture media and genetically manipulatable. These genetically manipulated *Burkholderia* cells can be experimentally infected into the host insect by oral feeding [5]. When host insect fitness and developmental rates were compared between insects harboring *Burkholderia* gut symbiont and *Burkholderia*-absent apo-symbiont insects, the beneficial consequences were observed on the host insect by the presence of gut symbiont [4]. These features of the *Riptortus–Burkholderia* gut symbiotic system provided good opportunities to study insect symbiosis at molecular and biochemical levels [6–15].

Bacterial peptidoglycan is an important factor to determine bacterial cell morphology [16]. Bacterial peptidoglycan hydrolyzing enzymes degrade the peptidoglycan by cleaving defined chemical bonds of the peptidoglycan [17]. These enzymes play an important roles in modulating bacterial cell growth and cell division, such as insertion of newly synthesized building blocks into nascent peptidoglycan, turnover of peptidoglycan and the splitting of daughter cells during cell division [17,18]. Among reported peptidoglycan hydrolases, *N*-acetylmuramyl-L-alanine amidases (Ami) are important for daughter cell separation in unicellular bacteria [19–21]. They cleave the amide bond between the L-Ala residue of the peptidoglycan stem-peptide and the *N*-acetylmuramic acid (MurNAc) residue of peptidoglycan glycan backbone. Because *Escherichia coli* AmiABC triple mutant chain-formed cell

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morphology resembling filamentous bacteria [22], we tried to address how *Burkholderia* AmiC mutants affect the colonization of gut symbiont on the *Riptortus–Burkholderia* symbiotic system.

Here, we show that a deletion mutation in *Burkholderia amiC* gene affects cell separation, bacterial motility and symbiotic association with host insect. Our findings suggest that cell of *Burkholderia* gut symbiont is an important molecular factor for the host–bacteria symbiotic association.

## 2. Materials and methods

### 2.1. Bacteria, culture media, and reagents

Bacterial strains and plasmids used in the present study are listed in Table 1. *E. coli* cells were cultured at 37 °C in LB medium (1% tryptone, 0.5% yeast extract, and 0.5% NaCl). Cells of *Burkholderia* symbiont strain RPE75, a spontaneous rifampicin-resistant mutant derived from RPE64 [23], were cultured at 30 °C in YG medium (0.5% yeast extract, 0.4% glucose, and 0.1% NaCl). Antibiotics were used at the following concentrations unless otherwise described: kanamycin at 50 μg/ml and rifampicin at 30 μg/ml.

### 2.2. Construction of ΔamiC mutant

The deletion of the chromosomal *amiC* gene from the *Burkholderia* strain RPE75 was accomplished by allelic exchange and homologous recombination using a suicide vector pK18mobsacB containing the 5′ (*amiC*-L) and 3′ (*amiC*-R) regions of *amiC* gene by following the method reported previously [8]. The wild-type *Burkholderia* symbiont strain RPE75 was subjected to PCR using the primers *amiC*-L-P1 (5′-ACA CCA GGA TCC CGT TGC CCG CCT TCT GCG CGA-3′) and *amiC*-L-P2 (5′-CAC CTA TCT AGA CGC ATG CGC GAG CCG GGG CGC-3′) for the *amiC*-L region, and the primers *amiC*-R-P1 (5′-ACA CCA TCT AGA CTC GTG GAG ACG GCG TTC ATC-3′) and *amiC*-R-P2 (5′-CAC CTA AAG CTT CGG ACA CCG AAA CGG GCA ATA-3′) for the *amiC*-R region. PCR products and the pK18-mobsacB vector were digested with proper restriction enzymes, ligated, and transformed into *E. coli* DH5α cells. The transformed *E. coli* cells were selected on LB-agar plates containing kanamycin.

**Table 1**
Bacterial strains and plasmids used in this study.

| Strain and plasmid | Relevant characteristics[a] | Source or reference |
|---|---|---|
| *Strains* | | |
| *Burkholderia* symbionts | | |
| RPE75 | *Burkholderia* symbiont (RPE64); $Rif^r$ | [23] |
| BBL021 | RPE75 Δ*amiC*; $Rif^r$ | This study |
| BBL121 | BBL021/pBL21, complementation of *amiC*; $Rif^r$, $Km^r$ | This study |
| NM1 | RPE75 Tn-inserted no-motility mutants; $Rif^r$, $Km^r$ | [24] |
| BBL122 | Δ*fliC*/pBL22, complementation of *fliC*; $Rif^r$, $Km^r$ | This study |
| *Escherichia coli* | | |
| DH5α | φ80d*lacZ*ΔM15, *recA*1, *endA*1, *gyrA*96, *thi*-1, *hsdR*17 ($r_K-$, $m_K+$), *supE*44, *relA*1, *deoR*, Δ(*lacZYA-argF*) U169, *phoA* | Toyobo |
| HBL1 | PIR1 carrying pSTV28 and pEVS104; $Cm^r$, $Km^r$ | [8] |
| *Plasmids* | | |
| pEVS104 | oriR6K helper plasmid containing conjugal *tra* and *trb*; $Km^r$ | [33] |
| pK18mobsacB | pMB1ori allelic exchange vector containing oriT; $Km^r$ | [34] |
| pBBR122 | Broad host vector; $Cm^r$, $Km^r$ | [35] |
| pBL21 | pBBR122 derivative containing *amiC*; $Km^r$ | This study |
| pBL22 | pBBR122 derivative containing *fliC*; $Km^r$ | This study |

[a] $Rif^r$, rifampicin resistance; $Km^r$, kanamycin resistance; $Cm^r$, chloramphenicol resistance.

Positive colonies carrying a vector with the correct insert were further selected by colony PCR using the primer *amiC*-L-P1 and the vector primer aphII (5′-ATC CAT CTT GTT CAA TCA TGC G-3′). Donor *E. coli* cells carrying the pK18mobsacB containing *amiC*-L and *amiC*-R were mixed with recipient *Burkholderia* RPE75 cells and also *E. coli* HBL1 cells carrying a helper plasmid pEVS104 to transfer the cloned vector to the RPE75 cells. After allowing a single crossover by culturing cell mixtures of triparental conjugation on YG-agar, RPE75 cells with the first crossover were selected on YG-agar containing rifampicin and kanamycin. Positive colonies with the genomic integration of vector DNA were confirmed by PCR using the chromosomal primer *amiC*-up (5′-ATC GTC AGA TAC GAG ATG CAG CG-3′) and the vector primer aphII. The second crossover was allowed by culturing cells with the single crossover in YG media and *Burkholderia* cells with a double crossover were selected on YG-agar containing rifampicin and sucrose (200 μg/ml). The mutant strain with deletion of the *amiC* gene (BBL021) was identified by PCR using the primers *amiC*-up and *amiC*-down (5′-CTC AGG CAA CTT TGA CGC AC-3′) and sequencing of the PCR product.

### 2.3. Generation of ΔamiC/amiC complemented mutant

A DNA fragment containing the open reading frame of *amiC* gene was amplified from RPE75 strain using the primers *amiC*-com-P1 (5′-AGC CCG ACT TAC ACA CTC GT-3′) and *amiC*-com-P2 (5′-CGG TGA TCG GAC TCG TCT-3′). The amplified DNA fragment was cloned into the *Dra*I site of pBBR122 to generate the plasmid pBL21. The cloned plasmid was introduced into *E. coli* DH5α cells to generate donor cells. By triparental conjugation with the BBL021 recipient cells and *E. coli* HBL1 helper cells, the pBL21 plasmid carried by the donor *E. coli* DH5α cells was transferred to the recipient *Burkholderia* BBL021 cells, yielding the complemented *Burkholderia* BBL121 cells. The complemented mutant strain was selected on YG-agar with rifampicin and kanamycin.

### 2.4. Generation of ΔfliC/fliC complemented mutant

We have constructed Δ*fliC*/*fliC* complemented mutant using flagellin (FliC)-deficient Δ*fliC* mutant [24], which was provided by Dr. Kikuchi of AIST of Japan. A DNA fragment containing the open reading frame of *fliC* gene was amplified from RPE75 using the primers *fliC*-com-P1 (5′-CGC TTC CGG TGT ATC TCC A-3′) and *fliC*-com-P2 (5′-GAT CGT GTG AAA TTG GGC GG-3′). The amplified DNA fragment was cloned into the *Dra*I site of pBBR122 to generate the plasmid pBL22. The cloned plasmid was introduced into *E. coli* DH5α cells to generate donor cells. By triparental conjugation with the Δ*fliC* mutant recipient cells and *E. coli* HBL1 helper cells, the pBL22 plasmid carried by the donor *E. coli* DH5α cells was transferred to the recipient *Burkholderia* Δ*fliC* mutant cells, yielding the complemented *Burkholderia* BBL122 cells. The complemented mutant strain was selected on YG-agar with rifampicin and kanamycin.

### 2.5. Insect rearing and symbiont inoculation

*R. pedestris* were reared in our insect laboratory at 28 °C under a long day regime of 16 h light and 8 h dark as described previously [8]. When nymphal insects reached adulthood, the insects were transferred to a bigger container in which soybean plant pots were placed for food and cotton pads were attached to the walls as a substrate for egg laying. Eggs were collected daily and transferred to new cages for hatching. Newly molted second instar nymphs were provided with wet cotton balls soaked with a symbiont inoculum solution consisting of exponential phase *Burkholderia* cells suspended in DWA at a concentration of $10^7$ cells/ml. The care

and treatment of *Burkholderia* cells and insects in all procedures strictly followed the guidelines of the Pusan National University (PNU) Institutional Animal Care and Use Committee (IACUC) and the Living Modified Organ (LMO) Committee.

### 2.6. Colony forming unit (CFU) assays

Gut symbiont cells of the M4 midgut regions dissected from second instar *R. pedestris* nymphs were collected in 50 μl of 10 mM phosphate buffer (PB, pH 7.0) and homogenized by a pestle as described previously [8].

### 2.7. Measurement of bacterial growth in liquid media

Growth curves of the *Burkholderia* symbiont strains were examined either in YG medium or in M9 minimal medium (0.6% $Na_2HPO_4{\cdot}2H_2O$, 0.3% $KH_2PO_4$, 0.1% $NH_4Cl$, 0.05% NaCl, 0.0003% $CaCl_2$, 1 mM $MgSO_4$, 0.2% glucose). The starting cell solutions were prepared by adjusting the optical density at 600 nm ($OD_{600}$) to 0.02 in either YG medium or minimal medium using primary culture grown in YG medium at 30 °C for 18 h or 27 h. The cell solutions were incubated on a rotator shaker at 180 rpm at 30 °C for 36 h, whose $OD_{600}$ was monitored every 3 h using a spectrophotometer (Shimatzu, Japan) [11].

### 2.8. Motility assays on soft agar

Motility of the *Burkholderia* symbiont strains was measured their swimming radius in YG containing 0.3% agar concentration. Cells were grown to an $OD_{600}$ of 0.6 and 3 μl of each strain were inoculated in the center of YG plates. Plates were grown at 30 °C for 26 h and the diameter of bacterial migration circle was measured.

### 2.9. Histological observation and measurement of single cell proportion of Burkholderia strains

Equal numbers of exponentially growing cells suspended in 5 μl of medium were spotted on the slide glass and heat-fixed and stained with 1% crystal violet solution. The images of bacterial cells were observed by a light microscope using oil immersion lens (BX40; Olympus). To estimate the proportion of single cell in the *Burkholderia* cell culture, the culture solutions were initially measured for their $OD_{600}$. The same $OD_{600}$ value of bacterial solution was filtered using micro-filter device (5 μm pore size) and the $OD_{600}$ of the filtered solution was measured again.

## 3. Results

### 3.1. Symbiotic colonization defect of ΔamiC mutant Burkholderia strain

To study the function of AmiC, *N*-acetylmuramyl-L-alanine amidase, of *Burkholderia* symbiont and its role in symbiosis with *Riptortus* host, *Burkholderia amiC* mutant and Δ*amiC*/*amiC* complemented mutant strains were constructed. To address the in vivo symbiotic property of *Burkholderia* Δ*amiC* mutants, *Riptortus* insects were orally administered with Δ*amiC* mutant, Δ*amiC*/*amiC* complemented and wild-type *Burkholderia* strains with inoculum titer of $10^7$ cells/ml. At 36 h post oral infection, bacterial colonization rates in the symbiotic organ were assessed by CFU assay. In contrast to the colonization of wild-type strain (~$10^5$ - CFUs/insect), Δ*amiC* mutant exhibited no colonization in the host midgut. However, Δ*amiC*/*amiC* complemented *Burkholderia* strain restored the colonization rate as that of wild-type strain

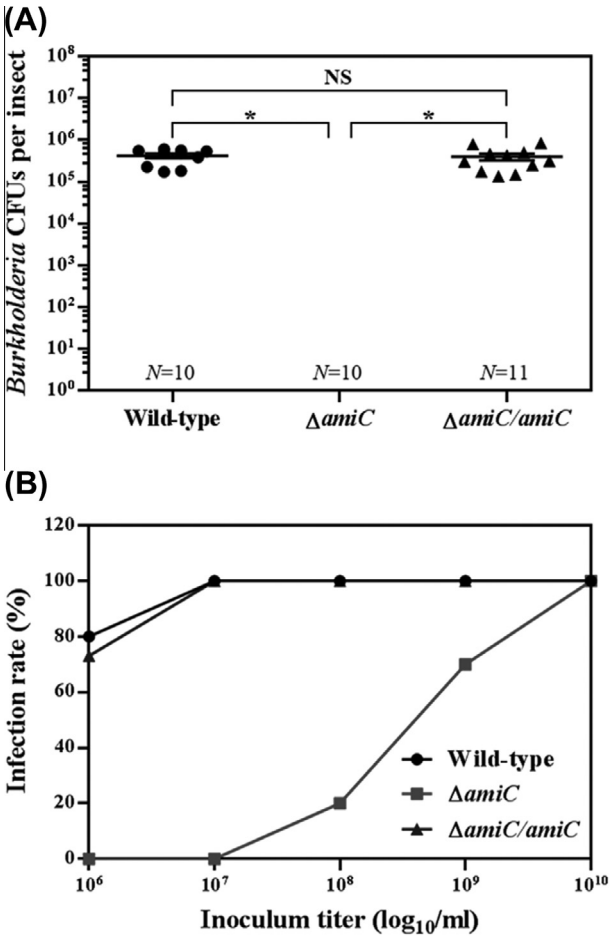


**Fig. 1.** (A) CFUs of the colonized *Burkholderia* and (B) infection rates after oral administration of wild-type (RPE75), Δ*amiC* (BBL021), and Δ*amiC*/*amiC* complemented *Burkholderia* strain (BBL121) (unpaired *t* test; $^*P < 0.0001$).

(Fig. 1A). When we infected with 1000 fold higher inoculum titer ($10^{10}$ cells/ml), colonization rate of Δ*amiC* mutant showed the same levels with those of wild-type and Δ*amiC*/*amiC* complemented *Burkholderia* strains (Fig. 1B). Once the Δ*amiC* mutant cells colonized the midgut, they continuously persist in the symbiotic midgut (data not shown). Based on these results, we assumed that Δ*amiC* mutant cells may have difficulty in physically reaching and survival inside the symbiotic host midgut.

### 3.2. In vitro characterization of ΔamiC mutant

When growth curves of the wild-type *Burkholderia* and the Δ*amiC* mutant strains were examined in nutritionally rich yeast-glucose (YG) medium, similar growth rates were shown, but the Δ*amiC*/*amiC* complemented strain grew slower than other two strains (Fig. 2A). Furthermore, although growth curves in nutritionally limited M9 minimal medium exhibited similar patterns, growth rates of three strains in minimal medium were slower than those in YG medium (Fig. 2B). These results indicate that deletion of the *amiC* gene does not affect the growth of the *Burkholderia* symbiont in vitro. The slower growth of the Δ*amiC*/*amiC* complemented mutant may be due to a cost of harboring the *amiC* plasmid. In contrast to the normal growth of the wild-type strain, Δ*amiC* mutant exhibited defect in cell motility examined by soft agar plates (Fig. 2C). The motility level of Δ*amiC* mutant was only 24% of the motility of wild-type strain (Fig. 2D). The motility defect of Δ*amiC* mutant was restored by expression of the functional *amiC*

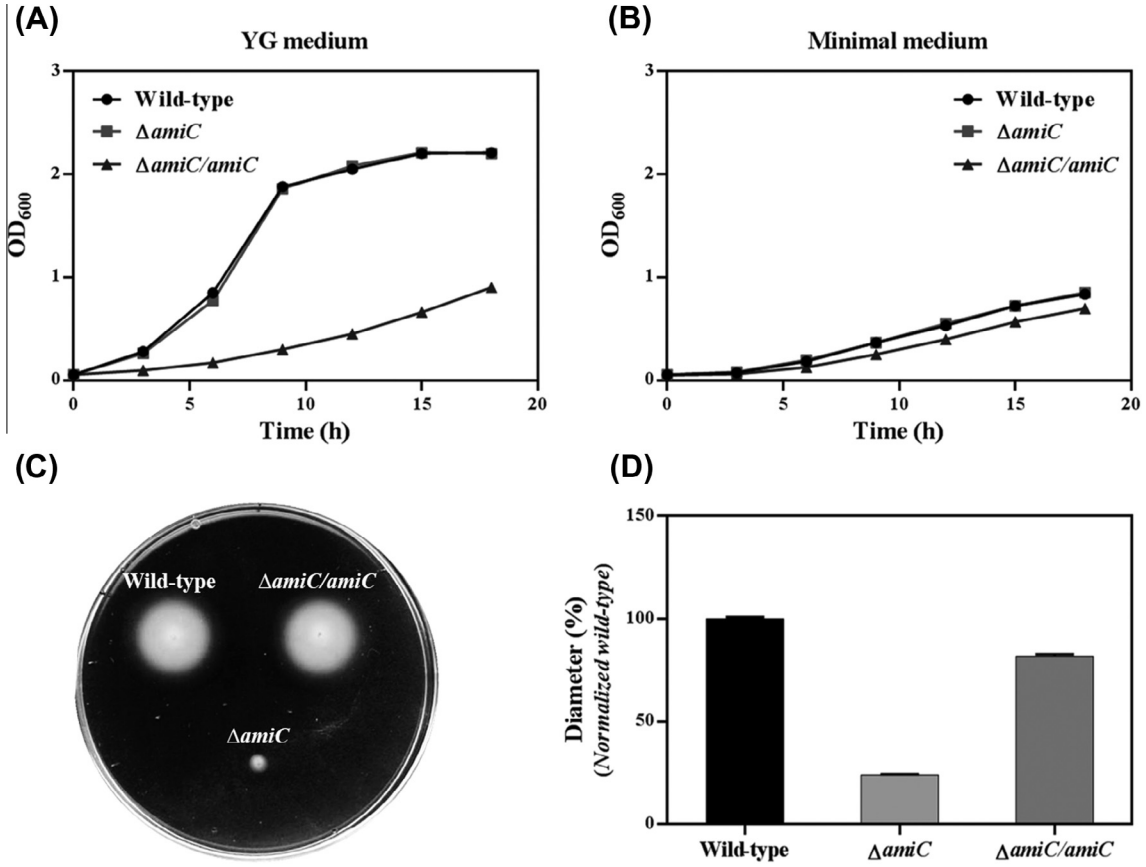


**Fig. 2.** (A) and (B) indicate that growth curves of the wild-type (RPE75), Δ*amiC* (BBL021), and Δ*amiC*/*amiC* complemented mutant *Burkholderia* strain (BBL121) in YG medium and in the nutrient minimal M9 medium, respectively. (C) Motility of indicated strains in YG plate containing 0.3% agar and (D) motility levels of indicated strains. Error bars indicate standard error of the mean.

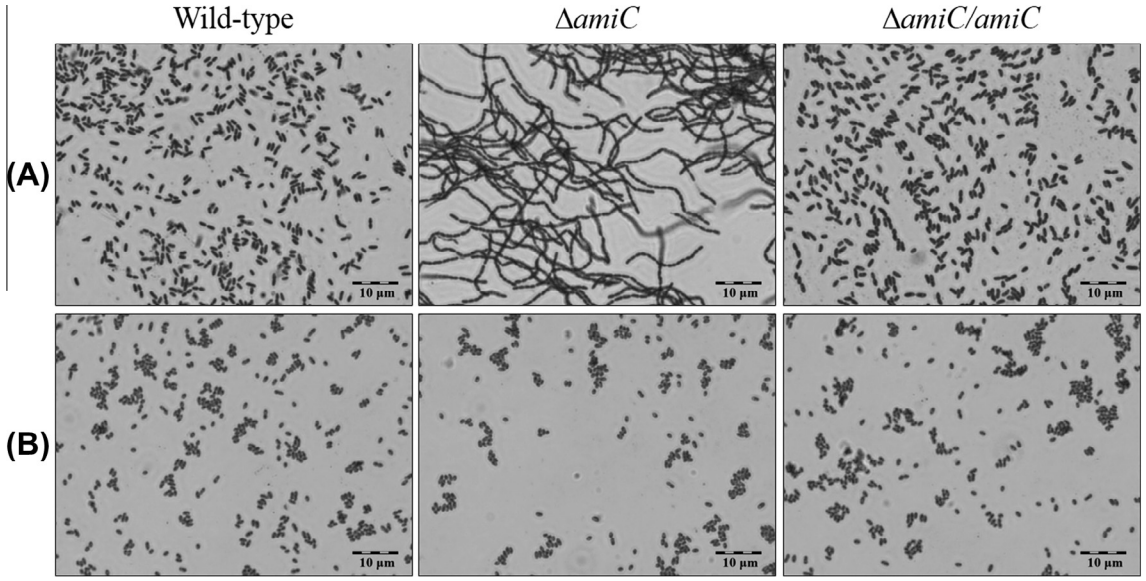


**Fig. 3.** (A) and (B) indicate light micrographic images of indicated strains cultured in YG medium and cultured in the nutrient minimal M9 medium, respectively.

gene, indicating that *amiC* mutation is solely responsible for the motility defect. Taken together, in vitro characterization of Δ*amiC* mutant suggests that the symbiotic colonization failure of Δ*amiC* mutant may be due to their motility defect, leading to inability to reach the symbiotic midgut.

### 3.3. Filamentous morphology of ΔamiC mutant

To address why Δ*amiC* mutant has motility defect, we examined the cell morphology of the Δ*amiC* mutant cells. The light microscopic image of the stained Δ*amiC* mutant cells showed

long-chained cell shape (Fig. 3A). The Δ*amiC*/*amiC* complemented *Burkholderia* strain showed single-celled morphology similar to the wild-type strain (Fig. 3A). But, interestingly, we observed that the filamentous morphology of Δ*amiC* mutant converted to the normal single-celled morphology by culturing them in M9 minimal medium (Fig. 3B). The different morphology of the Δ*amiC* mutant cells in different culture conditions suggests that AmiC may play a dominant role in cell separation in the nutrient-rich culturing condition, but, in the nutrient-limited condition, other amidase(s) may function in *Burkholderia* cell separation.

### 3.4. Motility of gut symbiont with single-celled phenotype is required for the colonization on host insect gut

To confirm the bacterial cell separation leading to generation of single-celled morphology is essential for establishing symbiosis, the single-celled Δ*amiC* mutant cells were examined for their symbiotic colonization ability. We first quantitatively assessed the single cell formation of Δ*amiC* mutant cells by culturing in M9 minimal medium. Bacterial culture solution ($OD_{600}$ = 0.6) containing both single-celled shape and filamentous cells was filtered through 5 μm pore filter to collect the single-celled bacteria. The $OD_{600}$ values of filtered bacterial solutions of the wild-type and Δ*amiC*/*amiC* complemented mutant strains showed almost same values as those of unfiltered solutions (columns 1, 2 and 7, 8, respectively, in Fig. 4A). However, Δ*amiC* mutant solution drastically decreased the $OD_{600}$ value after filtration because the chain-shaped cells were unable to pass through the 5 μm filter (columns 3 and 4). Expectedly, the $OD_{600}$ value of Δ*amiC* mutant cells cultured in the minimal media revealed the similar $OD_{600}$ value to the unfiltered solution (columns 5 and 6), verifying that cultivation in minimal media induces single-celled morphology of Δ*amiC* mutant.

When single-celled Δ*amiC* mutant cells cultured in minimal media were used to orally infect the *Riptortus* host, they were able to normally colonize the host midgut (columns 1 and 2 in Fig. 4B), showing that the bacterial density in the midgut were similar between the wild-type and the single-celled Δ*amiC* mutant cells. These results indicate the importance of single-celled shape of *Burkholderia* symbiont in symbiotic association with host *Riptortus*. However, because the filamentous cell morphology was assumed to be an artifact of growth in an environment that is not encountered in the insect host, we obtained a flagellin-deficient *Burkholderia* mutant strain (Δ*fliC*), which was recently characterized as a non-motile *Burkholderia* mutant [24]. Also, we constructed Δ*fliC*/*fliC* complemented mutant strain based on this Δ*fliC Burkholderia* mutant (Supplementary Fig. S1), and then examined whether the colonization defect of the filamentous *Burkholderia* forms was caused by the bacterial motility defect. When *Burkholderia* Δ*fliC* mutant and Δ*fliC*/*fliC* complemented mutant strains were used to orally infect the *Riptortus* host, *Burkholderia* Δ*fliC* mutant cells were unable to colonize the host midgut normally (column 3 in Fig. 4B), but Δ*fliC*/*fliC* complemented mutant colonized normally in the insect gut (column 4). These results clearly suggested that the motility of *Burkholderia* gut symbiont with single-celled phenotype is required for the colonization on insect gut.

## 4. Discussion

*Burkholderia* symbiont strain RPE75 is a unicellular rod-shape bacterium. To investigate the effect of bacterial cell morphology and cell motility onto the symbiotic association, we generated *Burkholderia* mutant deficient of a peptidoglycan hydrolase, AmiC. Peptidoglycan amidases remove the stem peptide from the glycan strands of peptidoglycan and break its cross-linked structure, leading to the severe defect in cell separation and formation of long-chained cells [18,25]. In our study, the *Burkholderia* Δ*amiC* mutant also formed the long chains of cells (Fig. 3A). However, the deletion of *amiC* gene in *Burkholderia* genome did not affect the bacteria cell growth in vitro (Fig. 2A and B), suggesting that disruption of *amiC* gene only induces the defect of cell separation but not affects the reproduction of daughter cell. In addition, when inoculated with high inoculum titer (~$10^{10}$ cells/ml), infection rate of Δ*amiC* mutant reached to the level of the wild-type strain (Fig. 1B). It is conceivable that the rarely present single cells or short-chained cells may exist in the very high titers of inoculum solution, could reach to the symbiotic organ and establish the symbiotic

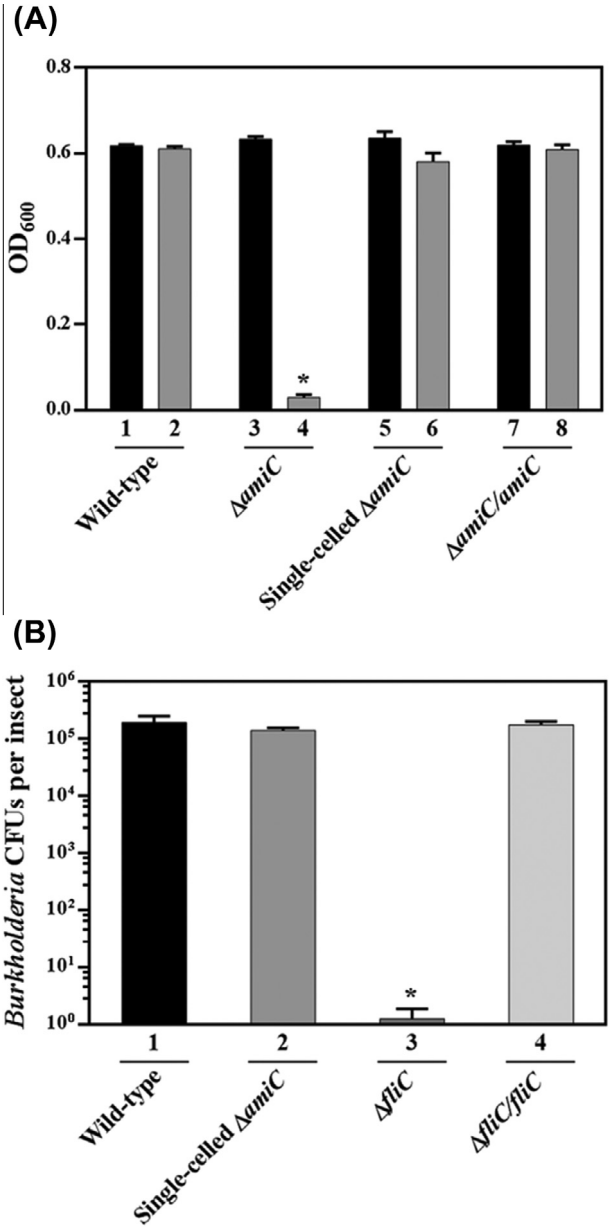


**Fig. 4.** (A) Quantification of proportion of single cells before (black bars) and after (gray bars) filtration through 5 μm filter. Columns 1, 2 and 3, 4 and 5, 6 and 7, 8 indicate *Burkholderia* wild-type (RPE75), Δ*amiC* (BBL021), single-celled Δ*amiC*, and Δ*amiC*/*amiC* complemented *Burkholderia* strain (BBL121), respectively. (B) CFU quantification of gut-colonized *Burkholderia* wild-type (RPE75, column 1), single-celled Δ*amiC* (column 2), Δ*fliC* (column 3) and Δ*fliC*/*fliC* mutant (column 4). (unpaired *t* test; $^{*}P < 0.0001$, $N = 10$). Error bars indicate standard error of the mean.

association. Interestingly, we found that cell morphology of bacteria could be converted by culturing condition. The filamentous Δ*amiC* mutant in nutritionally rich medium was changed to single-celled morphology in minimally limited medium (Fig. 3B). *Burkholderia* genome has three peptidoglycan hydrolyzing amidase genes and these gene products may attribute to the redundancy of amidase function in the nutrient-limited condition. We speculate that AmiC may be a dominant amidase in nutrient-rich culturing condition since a single Δ*amiC* mutation resulted in formation of chains of cells. However, other amidases may expressed and/or play a redundant role in cell separation in the nutrient-limited condition. In previously reports, some bacteria can modify their morphology in response to environmental cues or during the course of pathogenesis [26], indicating that cell morphology is important for bacterial adaptation.

A rod shaped cell becomes small and round to conserve energy during nutritional scarcity and prevent capture by predators [26,27]. Cyanobacteria change their morphology to cope with different environmental variability and/or stresses and to maximize utilization of available resources, including light and nutrients [28]. Carbon, phosphorus, nitrogen, and iron were suggested as the bacterial morphology or growth-changing factors [29]. Modifications of cellular morphology in response to nutrient limitation probably allow bacteria to more fully utilize or mobilize the available resources to support their survival, growth and reproduction [28]. As shown in Fig 3, the morphology of *Burkholderia* cell cultured in minimal medium exhibited the circle-shape and smaller size than those cultured in YG medium, suggesting that the limitation of nutrients in minimal media affect the *Burkholderia* cell morphology.

The previous studies showed that bacterial motility is required for the symbiotic association with host [30,31]. Motility of *Vibrio fischeri* cells is important to specifically colonize the light-emitting organ of their host, the squid *Euprymna scolopes* [30,31] and motility of entomopathogenic *Photorhabdus luminescens* is required for the competitive fitness during insect infection [32]. Therefore, to address a possibility of that the filamentous cell morphology was due to the artifact of growth in an environment, we obtained a flagellin-deficient *Burkholderia* mutant strain (Δ*fliC*), which was recently reported as a non-motile *Burkholderia* mutant strain [24]. By using the additional *Burkholderia* Δ*fliC* mutant strain, we clearly addressed the reasons of why un-motile filamentous non-motile bacteria have failed to associate with host, suggesting that motility of *Burkholderia* symbiont is important for the symbiotic association with *Riptortus* host insect.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2015.08.022.

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